44. The method of any one of claims 37-41, wherein binding of said test compound to said polypeptide is detected by the use of a competition binding assay.

45. The method of any one of claims 37-41, wherein said test compound modulates the activity of said polypeptide.

REMARKS

Claims 1-26 were pending in the application. Claims 1-19 and 22-26 have been cancelled without prejudice, as being directed to a non-elected invention. Applicant reserves the right to traverse the restriction between the non-elected Groups in this or a separate application. Claims 20 and 21 have also been cancelled and new claims 27-45 have been added. Accordingly, after the amendments presented herein have been entered, claims 27-45 will be pending. For the Examiner's convenience all of the pending claims are set forth in Appendix A.

Support for the new claims can be found throughout the specification and the claims as originally filed. Specifically, support for claims 27-30 and 37-40 can be found in claims 20 and 21 as originally filed. Support for new claims 31 and 41 can be found at page 5, lines 7-10 of the specification. Support for new claims 32-34 can be found at page 45, lines 7-17 of the specification. Support for new claim 35 can be found at page 45, lines 7-10 and page 27, lines 30-32 of the specification. Support for new claim 42 can be found at page 48, line 32 through page 49, line 5 of the specification. Support for new claim 43 can be found at page 45, line 33 through page 46, line 18 of the specification. Finally, support for new claim 44 can be found at page 46, lines 11-16 of the specification.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendments. The attached page is captioned "Version With Markings to Show Changes Made".

At paragraph 3 of the present Office Action, the Examiner has requested that a paper copy of the sequence listing be submitted by the Applicant. Applicant respectfully submits that, pursuant to 37 C.F.R. § 1.821 (e), the Examiner was authorized to use the paper and computer

readable forms of the Sequence Listing of the parent application Serial No: 09/439,165. Nonetheless, for the Examiner's convenience, a paper copy of the sequence listing is being submitted herewith.

No new matter has been added. Any cancellation of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely to expedite the prosecution of the application. Applicant reserves the right to pursue the claims as originally filed in this or a separate application(s).

Objection to Claims 20 and 21

The Examiner has objected to claims 20 and 21 as being "of improper dependent form for failing to further limit the subject matter of a previous claim."

Applicant has cancelled claims 20 and 21, thus, rendering this objection moot. In view of the foregoing, Applicant respectfully requests that the Examiner reconsider and withdraw this objection.

Objections to the Disclosure

The Examiner has objected to the numbering of Figure 1 because, "partial views of a drawing which are intended to form one complete view must be identified by the same number followed by a capital letter."

Applicant respectfully submits that the Figures and the Brief Description of the Drawings have been amended according to the Examiner's request. A substitute copy of Figure 1, as amended, is submitted herewith. In view of the foregoing, Applicant respectfully requests that the Examiner reconsider and withdraw the foregoing objection to the disclosure.

Rejection of Claims 20 and 21 Under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected claims 20 and 21 under 35 U.S.C. § 112, first paragraph because, according to the Examiner, "the specification, ***while being enabling for the practice of***

a method of identifying a ligand which binds to a receptor protein comprising the amino acid sequence presented in SEQ ID NO:5 of the instant specification, does not reasonably provide enablement for the practice of a binding assay which employs a protein having anything less than the entire amino acid sequence presented in SEQ ID NOs:5, 11 or 20." (***Emphasis added***).

In particular, the Examiner states that

> [b]ecause the instant specification does not identify those amino acid residues in the amino acid sequence of SEQ ID NO:5 which are essential for biological activity and structural integrity of a h VR-2 protein and those residues which are either expendable or substitutable, a practitioner can not make and use a polypeptide lacking that entire sequence with any reasonable expectation that the altered protein would respond in an authentic manner. In the absence of such structure-function information a practitioner would have to resort to a substantial amount of undue experimentation in the form of insertional, deletional and substitutional mutation analysis of over 760 amino acid residues before they could even begin to rationally design an hVR-2 polypeptide having other than a natural amino acid sequence and which responds in a binding assay in a manner which is representative of the native protein.

Applicant respectfully traverses the foregoing rejection for the following reasons.

The pending claims are directed to methods for identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NOs:5 or 20 or a fragment of the amino acid sequence of SEQ ID NO:5 by :i) contacting the polypeptide, or a cell expressing the polypeptide with a test compound; and ii) determining whether the polypeptide binds to the test compound.

With respect to the claimed methods that use the entire polypeptide of SEQ ID NOs:5 or 20, Applicant respectfully submits that the Examiner has admitted that the specification is "enabling for the practice of a method of identifying a ligand which binds to a receptor protein comprising the amino acid sequence presented in SEQ ID NO:5" (see page 3 of the present Office Action). Accordingly, Applicant respectfully requests that the Examiner indicate as allowable claims 27-30 and 37-40, (and claims depending therefrom) which are directed to methods that use the entire polypeptide sequence of SEQ ID NOs:5 or 20.

With respect to the claimed methods that use fragments of the polypeptide of SEQ ID NO:5, Applicant respectfully submits that based on the teachings in Applicant's specification,

and the knowledge generally available in the art at the time of filing, the skilled artisan would be able to practice the claimed methods using only routine experimentation.

To begin with, Applicant has taught in the instant specification which regions of the VR-2 molecules are important for activity and, thus, which regions of the molecule would respond in a binding assay in a manner which is representative of the native protein. Specifically, as taught in the specification (at, for example, page 10, lines 1-35), the VR-2 polypeptide contains ankyrin repeats, transmembrane domains, and at least one proline rich domain, all characteristic and necessary for the function of the Capsaicin/Vanilloid family of receptors. Based on these teachings the skilled artisan would be equiped to use a fragment containing one or more of these domains to identify compounds that bind to a domain and modulate an activity of the Capsaicin/Vanilloid receptor. Compounds identified in such a manner would be of practical value, *e.g.*, they could be used as antagonists of a Capsaicin/Vanilloid receptor. For example, a fragment of a VR-2 polypeptide that contains the proline rich domain may be used by the skilled artisan to identify compounds capable of binding the proline rich domain and disrupting interactions with SH3 domain-containing proteins that act downstream of the VR-2 receptor in a pain signaling pathway. Such a compound would be expected to inhibit the function of the VR-2 polypeptide by disrupting the VR-2 signaling pathway.

As indicated by U.S. Patent Nos. 6,214,564 6,214,559, and 6,187,552 (copies of which are submitted herewith as Appendices B, C and D, respectively) fragments of proteins are typically used in screening assays, such as binding assays. In fact, the use of polypeptide fragments in such assays, instead of a full length protein molecule, may be preferable in some situations because of the ease in preparing and handling of the smaller polypeptide fragments. Screening assays utilizing polypeptide fragments are routinely used in the art and have resulted in the identification of a plethora of useful compounds (including a number of commercially available drugs).

Moreover, Applicant has taught how such fragments would be generated and used in binding assays. For example, at page 27, lines 5-28 of the specification, Applicant teaches methods for generating and screening fragments of VR-2 receptors. Specifically, Applicant teaches methods to generate a library of VR-2 fragments by creating double stranded DNA fragments that encode N-terminal, C-terminal and internal fragments of various sizes. Applicant

further teaches how a skilled artisan would insert these fragments into an expression vector and how one would assay the resulting VR-2 fragments. Based on these teachings in Applicant's specification the skilled artisan would be able to make and use fragments of the VR-2 receptor.

The Examiner further states that

> because the differences between the amino acid sequence of the hVR- 2 protein of the instant invention and that of hVR-1 are greater than the similarities, one would not conclude that these two proteins bind the same spectrum of ligands or modulate the same cellular processes.

Applicant respectfully traverses this rejection for the following reasons. Applicant has shown a sequence alignment of human VR-1 (SEQ ID NO:2) and human VR-2 (SEQ ID NO:5) that shows amino acid identity between the two polypeptides. Both polypeptides are characterized by the same biologically active domains, *e.g.,* ankyrin repeats, transmembrane domains, and at least one proline rich domain, and both polypeptides are expressed in the same type of trigeminal sensory neurons known to be involved in nociception. Applicant has also taught in the specification that the claimed VR-2 molecules are part of a calcium regulation system involved in pain response. Based on these teachings an ordinary skilled artisan would conclude that the claimed receptor is, indeed, a capsaicin/vanilliod receptor belonging in the same family as the hVR-1 receptor.

The Examiner is also of the opinion that

> the instant specification does not provide a method through which an artisan can determine if a derivative of the disclosed protein retains functionality because the instant specification discloses neither a demonstrated ligand for the instant receptor or the pathway through which it has been shown to signal. A receptor, by definition, must bind a ligand and transduce a signal. To determine if a derivative of a receptor has retained its function an artisan must be able to measure both of these activities. Since the instant specification does not identify a ligand which has been demonstrated to bind to the disclosed protein, it is not possible to determine if ligand-binding is retained.

Contrary to the Examiner's assertions the identity of the natural ligand of the VR-2 receptor proteins described in the specification is not requisite to the enablement of the claimed nucleic acid molecules. For example, Silverman *et al.* (1998) and U.S. Patent No. 6,110,693, (provided herewith as Appendices E and F, respectively), describe methods of assaying GPCR activity *without knowledge of the receptor ligand.* Modulators of VR-2 activity can be identified by one of skill in the art without knowledge of the natural ligand through methods known in the art, such as, for example, the methods described in the references cited above, *e.g.,* the high-throughput cell-based methods described in Silverman *et al.*

The Examiner states that the claims

> encompass an assay which requires a practitioner to measure VR -2 activity in response to the binding of a ligand to that receptor. To practice the claimed assay one must be able to measure a signal which is transduced by the ligand activation of an hVR-2 protein and the instant application does not identify a specific cellular signal which has been shown to be modulated by the binding of a ligand to a receptor of the instant invention

Applicant respectfully traverses this rejection for the following reasons. Contrary to the Examiner's assertions, Applicant has taught multiple methods for measuring binding of a ligand to a VR-2 receptor. For example, Applicant has taught methods for measuring changes in intracellular calcium levels and membrane depolarization, signals which are modulated by the binding of a ligand to a VR-2 receptor (see, for example, page 45, lines 10-14 or page 27, lines 29-37 of the specification). Applicant further teaches methods to measure binding of a ligand to a VR-2 receptor *in vitro*. At page 45, line 18 through page 48, line 16, Applicant teaches methods for measuring the interaction of a test compound with VR-2 receptor, *e.g.,* coupling the VR-2 substrate with an enzymatic or radioisotopic label, measuring the cytosensor, and measuring association and disassociation using surface plasmon resonance. Based on these teachings in the Applicant's specification an ordinary skilled artisan would be able to measure the binding of a ligand to the claimed VR-2 receptors (*e.g.,* by measuring a signal that is generated by binding of a ligand to the receptor).

In view of the foregoing teachings in Applicant's specification and the knowledge generally available to the skilled artisan at the time of the invention, the skilled artisan would be able to practice the claimed invention using only routine experimentation. Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw the foregoing rejection.

Rejection of Claims 20 and 21 Under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected claims 20 and 21 under 35 U.S.C. § 112, first paragraph because, according to the Examiner, these claims contain "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention." Specifically the Examiner is of the opinion that "[t]here is absolutely no written description in the instant specification of an allelic variant of a protein comprising the amino acid sequence presented in SEQ ID NO:5 of the instant application."

In the interest of expediting prosecution, all claims drawn to allelic variants have been cancelled, thus, rendering this rejection moot. Accordingly, Applicant respectfully requests that the Examiner reconsider and withdraw the foregoing rejection.

The cancellation of these claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely to expedite the prosecution of the application. Applicant reserves the right to pursue the claims as originally filed in this or a separate application(s).

Rejection of Claims 20 and 21 Under 35 U.S.C. § 102(a) as Being Anticipated by Julius et al and Davis et al.

The Examiner has rejected claims 20 and 21 as being anticipated by Julius *et al.* (WO 99/37675) and by Davis *et al.* (WO 99/37765). The Examiner states that SEQ ID NO:5 of the instant application is identical to SEQ ID NO:36 of Julius *et al.* and SEQ ID NO:2 of Davis *et al.*

Applicant respectfully traverses this rejection for the following reason. The instant application claims and is entitled to priority to U.S. Patent Application Serial No: : 09/258,633 filed on ***February 26, 1999***. Julius *et al.* (WO 99/37675) and by Davis *et al.* (WO 99/37765) both published on ***July 29, 1999***. Accordingly, Applicant respectfully submits that the Julius *et al.* and Davis *et al.* references, ***which have an effective prior art date after the effective filing date of the pending application***, are not available as prior art against the present application and, thus, respectfully requests that the Examiner reconsider and withdraw this rejection.

Notwithstanding that Julius *et al.* and Davis *et al.* are not available as prior art, Applicant respectfully notes that the cited sequences are not identical to those of SEQ ID NO:5 of the instant application. A sequence alignment of SEQ ID NO:36 of Julius *et al.* and SEQ ID NO:5 of the instant application shows that there is a single amino acid insertion at residue 420 of SEQ ID NO:5 that is not present in SEQ ID NO:36. A sequence alignment of SEQ ID NO:2 of Davis *et al.* and SEQ ID NO:5 of the instant application shows multiple mismatches in the amino acid sequence between residues 310 and 565 of SEQ ID NO:5. For the Examiner's convenience a copy of both sequence alignments are provided herewith as Appendices G and H, respectively.

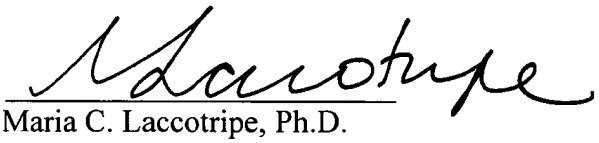
A sequence alignment of SEQ ID NO:36 of Julius *et al.* and SEQ ID NO:20 of the instant application shows multiple gaps and mismatches in the amino acid sequence beginning at residue 450 of SEQ ID NO:20. A sequence alignment of SEQ ID NO:2 of Davis *et al.* and SEQ ID NO:20 of the instant application shows an insertion of approximately 130 amino acid residues beginning at 533 of SEQ ID NO:20. For the Examiner's convenience a copy of both sequence alignments are provided herewith as Appendices I and J, respectively

SUMMARY

If a telephone conversation with Applicant's attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP



Maria C. Laccotripe, Ph.D.
Attorney for Applicant
Limited Recognition Under 37 CFR §10.9(b)

28 State Street
Boston, MA 02109
Telephone: (617) 227-7400
Facsimile: (617) 742-4214

Dated: January 31, 2002

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Figures 1A-D depicts the full length cDNA sequence and predicted amino acid sequence of human VR-1 (hVR-1). The nucleotide sequence corresponds to nucleic acids 1 to 3909 of SEQ ID NO:1. The amino acid sequence corresponds to amino acids 1 to 839 of SEQ ID NO:2. The coding region without the 5' and 3' untranslated regions of the human VR-1 (hVR-1) gene is shown in SEQ ID NO:3.

Appendix A

27. A method for identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:5, the method comprising:

a) contacting a cell expressing the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:5.

28. A method for identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:20, the method comprising:

a) contacting a cell expressing the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:20.

29. A method for identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:5, the method comprising:

a) contacting a cell expressing the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:5.

30. A method for identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:20, the method comprising:

a) contacting a cell expressing the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:20.

31. A method for identifying a compound which binds to a polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:5, the method comprising:

a) contacting a cell expressing the polypeptide with a test compound under conditions suitable binding; and

b) determining whether the test compound binds to said polypeptide, thereby identifying a compound which binds to a polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:5.

32. The method of any one of claims 27-31, wherein binding of the test compound to the polypeptide is detected by the use of an assay for a hVR-2 activity.

33. The method of claim 32, wherein said hVR-2 activity is modulation of membrane depolarization.

34. The method of claim 32, wherein said hVR-2 activity is modulation of intracellular calcium levels.

35. The method of any one of claims 27-31, wherein said cell expressing said polypeptide is a neuronal cell.

36. The method of any one of claims 27-31 , wherein said compound modulates the activity of said polypeptide.

37. A method for identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:5, the method comprising:

a) contacting a sample comprising the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:5.

38. A method for identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:20, the method comprising:

a) contacting a sample comprising the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:20.

39. A method for identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:5, the method comprising:

a) contacting a sample comprising the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:5.

40. A method for identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:20, the method comprising:

a) contacting a sample comprising the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to the polypeptide, thereby identifying a compound which binds to a polypeptide consisting of the amino acid sequence of SEQ ID NO:20.

41. A method for identifying a compound which binds to a polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:5, the method comprising:

a) contacting a sample comprising the polypeptide with a test compound under conditions suitable for binding; and

b) determining whether the test compound binds to said polypeptide, thereby identifying a compound which binds to a polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:5.

42. The method of any one of claims 27-31 or 37-41, wherein binding of said test compound to said polypeptide is detected by the use of a yeast two-hybrid assay.

43. The method of any one of claims 37-41, wherein binding of said test compound to said polypeptide is detected by the use of a direct binding assay.

44. The method of any one of claims 37-41, wherein binding of said test compound to said polypeptide is detected by the use of a competition binding assay.

45. The method of any one of claims 37-41, wherein said test compound modulates the activity of said polypeptide.

New assay technologies for high-throughput screening

Lauren Silverman, Robert Campbell and James R Broach*

The use of high-throughput screening for early stage drug discovery imposes several constraints on the format of assays for therapeutic targets of interest. Homogeneous cell-free assays based on energy transfer, fluorescence polarization spectroscopy or fluorescence correlation spectroscopy provide the sensitivity, ease, speed and resistance to interference from test compounds needed to function in a high-throughput screening mode. Similarly, novel cell-based assays are now being adapted for high-throughput screening, providing for *in situ* analysis of a variety of biological targets. Finally, recent advances in assay miniaturization mark a transition to ultra high-throughput screening, ensuring that identification of lead compounds will not be the rate-limiting step in finding new drugs.

Addresses
Cadus Pharmaceutical Corporation, 777 Old Saw Mill River Road, Tarrytown, NY 10591-6705, USA
*Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA; e-mail: jbroach@molecular.princeton.edu

Current Opinion in Chemical Biology 1998, 2:397–403

http://biomednet.com/cgcrel/1367593100200397

Abbreviations

CRE	cAMP response element
FCS	fluorescence correlation spectroscopy
GFP	green fluorescent protein
HTS	high-throughput screening

Introduction

Continuing advances in molecular biology, human genetics and genomics have accelerated identification of the mechanisms underlying a growing number of human diseases. This progress has increased the number of novel protein targets available for potential therapeutic intervention by drug treatment. Concurrently, novel approaches in combinatorial chemistry and expanded collections of natural products have dramatically increased the number of compounds that can be tested for activity against these targets. The confluence of these two trends towards more potential targets and larger chemical libraries has greatly stimulated adoption of high-throughput screening (HTS) as the primary tool for early stage drug discovery.

HTS is the process by which large numbers of compounds are tested, in an automated fashion, for activity as inhibitors or activators of a particular biological target, such as a cell surface receptor or a metabolic enzyme. Although any assay performed on the bench top can, in theory, be applied in HTS, conversion to an automated format imposes certain constraints that affect the design of the assay in practice. Procedures that are routine at the bench are often extremely difficult to automate. Also, the more steps required for an assay, the more difficult to automate the HTS. The ideal assay is one that can be performed in a single well with no other manipulation other than addition of the sample to be tested.

A number of assay formats have been developed or modified over the past few years to conform to the constraints imposed by HTS. These assay protocols can be divided into two groups: cell-free assays that measure the biological activity of a relatively pure protein target and cell-based assays that assess the activity of a target protein by monitoring a biological response of a cell in which the target protein resides. In either case, the protocols require minimal manipulations, can be performed robotically in relatively small volumes, yield robust responses and are relatively impervious to perturbation by solvents and compounds used in drug screening. In this review we describe several of the more recently developed or exploited assay protocols for HTS.

Cell-free assays

The primary goal in adapting cell-free assays to HTS is to minimize the number of steps required in setting up the assay and in detecting the activity, be it an enzymatic reaction or the binding of two components. This goal has been met to a large extent by development of detection systems that do not require separation of the product of the reaction from substrate, or from other components of the assay mixture. Earlier approaches to such homogeneous assay formats relied on proximity-dependent energy transfer. The output of such assays derived from the signal enhancement generated by bringing a source and a distance-dependent amplifier close together. For example, the β-particles of a low-energy radionuclide attached to a ligand will stimulate the fluorescent emission of a scintillant in a bead to which the ligand's receptor is attached [1,2]. More recently, this detection method has been applied to enzymatic reactions, such as that catalyzed by topoisomerase I [3]. As another example of energy transfer assay formats, the rare earth metal lanthanide, Eu^{2+}, when irradiated by light, can transfer its excitation energy in a nonradiative process to the fluorescent protein, allophycocyanin, if the two are in close proximity. This can occur when a Eu^{2+}-derivitized ligand binds to an allophycocyanin-linked receptor [4,5] or a Eu^{2+}-derivitized anti-phosphotyrosine antibody binds to a detector-linked phosphorylated substrate of a tyrosine kinase such as src [6•]. Use of time resolved fluorescent procedures assessing emission at specific times following excitation enhances the sensitivity of this technique by reducing interference from background fluorescence, from test compounds or from assay components [6•,7•]. Finally, enzymatic assays suitable for HTS and based on fluorescent resonant energy

transfer between two different forms of green fluorescent protein (GFP) have recently been described [8•].

A number of investigators have exploited fluorescence polarization spectroscopy (FPS) as the basis for homogeneous HTS assays of both enzymatic and binding reactions. When fluorescent molecules in solution are excited with polarized light, the degree to which the emitted light retains polarization depends on the extent to which the fluorescent molecule rotates during the interval between excitation and emission. The rapid rotation of small fluorescent molecules in solution results in substantial loss of polarization. If such small molecules bind to larger molecules, their rotational diffusion is reduced and the retention of polarization is correspondingly increased. Thus, by measuring the relative intensity of emitted light in the planes normal and orthogonal to the plane of the incident polarized light, the extent of rotation of a target molecule, and inferentially, the extent of binding of the target molecule to a larger component, can be calculated. For instance, fluorescent polarization has been used to detect the presence of specific drugs or hormones [9,10], to assess antibody binding of fluorescein-conjugated peptides [11] or to monitor DNA:DNA hybrid formation [12]. The recent availability of a 96-well plate reader [13] with a high sensitivity to fluorescein and fluorescein conjugates has allowed development of 96-well based fluorescent polarization assays. Such high-throughput assays for src family tyrosine kinase activity [14•], for binding of phosphopeptides to Src SH2 domains [15•], for interaction between STAT1 and an γ-interferon receptor-derived phosphotyrosine-containing peptide [16•] and for specific protease activities [17,18•] have recently been described. The sensitivity of fluorescence polarization, the ease and speed with which such assays can be run and the resistance of such assays to interference from absorptive compounds commonly present in complex mixtures [18•] make this procedure highly amenable to HTS.

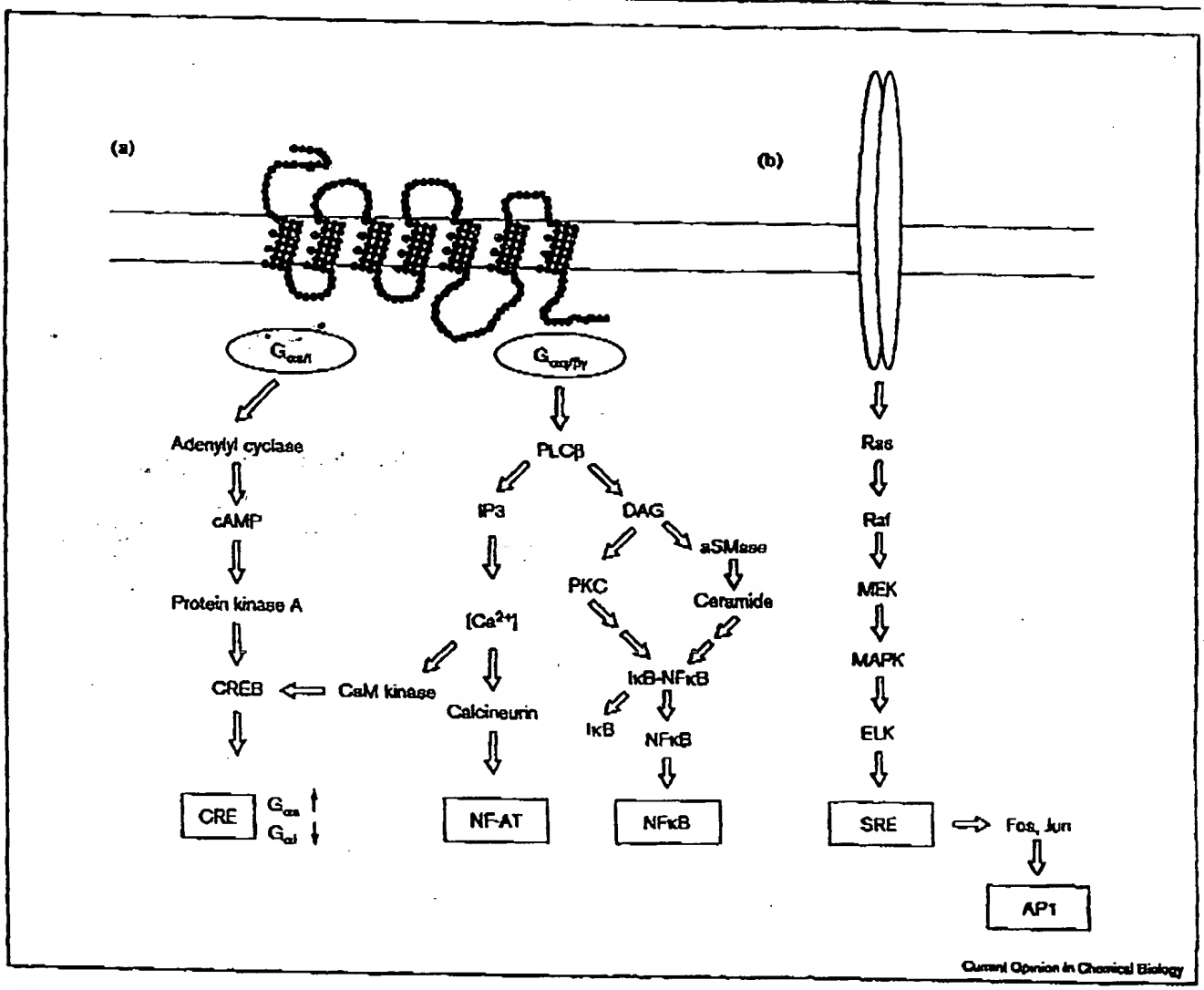
Fluorescence correlation spectroscopy (FCS) represents another recently developed detection format eminently suitable for HTS. FCS measures differences in physical states of a target molecule, such as bound versus free or cleaved versus intact, in a homogeneous mixture [19]. Specifically, FCS measures the burst of fluorescent emission of a molecule passing through a small volume of space, which is defined by a sharply focused laser beam. Small molecules diffuse through the volume rapidly and thus yield short bursts of light. Binding of these small molecules to larger molecules reduces their translational diffusion and correspondingly increases the duration of the bursts of light. Deconvolution of the emission patterns in a sample by appropriate software can yield the relative amount of the bound and unbound states of a fluorescently tagged ligand. This technology can therefore readily be applied to measure receptor–ligand interactions, DNA–protein interactions, nucleic acid hybrid formation and certain enzymatic reactions [20].

Cell-based assays

Cell-based assays are an increasingly attractive alternative to *in vitro* biochemical assays for HTS. Such *in vivo* assays require an ability to examine a specific cellular process and a means to measure its output. For instance, agonist activation of a cell surface receptor or a ligand-gated ion channel can elicit a change in the transcription pattern of a number of genes. This ligand-induced alteration in transcription can be readily captured by using gene fusions, in which a promoter element responsive to receptor activation is fused to the coding region for an enzyme or protein whose levels can be easily measured. Appreciation of the particular signaling pathway associated with a specific receptor allows identification of the appropriate transcriptional response element required to detect a response. Figure 1 depicts a number f signal transduction pathways, indicating the transcriptional response elements coupled to each pathway. Several reporter genes that generate products that can be adapted to HTS format are available [21,22]. These are listed in Table 1, with references to recent innovations in their use [23•,24,25,26•]. For instance, the recent report of novel fluorescent, cell-permeable substrates for β-lactamase documents the use of β-lactamase to detect receptor activation in single cells, making it an attractive assay system for high density HTS [27••].

While cell-based assays using reporter genes have proved effective as an HTS format, detecting more immediate responses to target protein activation provides several advantages, including shorter duration of the assay and fewer false positives from nonspecific interactions. As indicated in Figure 1, such cellular response dependent on activation of a receptor include elevation of a second messenger (for example, Ca^{2+}, cAMP, inositol trisphosphate), phosphorylation of an intermediate signaling protein, or subcellular translocation of a signaling molecule. Recent advances in molecular biology and in instrumentation have made it possible to monitor these events in an automated format. For instance, the recent availability of a 96-well fluorescent imaging plate reader (Molecular Devices, Sunnyvale, California, USA) permits HTS of receptor activation by monitoring Ca^{2+} mobilization of cells preloaded with a fluorescent calcium indicator, such as FLUO-3 (Molecular Probes, Eugene, Oregon, USA). In addition, recombinant cells expressing a calcium-sensitive fluorescent protein, such as aequorin [28•] or a hybrid calmodulin–GFP protein [29••], obviate the need for preloading cells with dyes in order to detect calcium fluxes following stimulation. A separate approach to detecting early events following receptor stimulation involves examining relocalization of specific components of the signal transduction machinery. For instance, MAP kinase (Figure 1) relocalizes from the cytoplasm to the nucleus within minutes following stimulation of an upstream G-protein-coupled receptor [30,31]. Similarly, Barak *et al.* [32•] have shown that recruitment of a β-arrestin–GFP fusion protein to the plasma membrane can be used to monitor activation

Figure 1



Signal transduction pathways commonly used in mammalian cell-based high-throughput assays. (a) Agonist-engaged seven transmembrane receptors are functionally linked to the modulation of several well characterized enhancer/promoter elements, the cAMP response element (CRE), nuclear factor of activated T cells (NF-AT), NFκB, serum response element (SRE) and AP1 [46–49]. Upon activation of a $G_{\alpha s}$ coupling receptor, adenylyl cyclase is stimulated, producing increased concentrations of intracellular cAMP, stimulation of protein kinase A, phosphorylation of the CRE binding protein (CREB) and induction of promoters with CRE elements. $G_{\alpha i}$ coupling receptors dampen CRE activity by inhibition of the same signal transduction components. $G_{\alpha q}$ coupling receptors and some βγ pairs stimulate phospholipase C (PLC), and the generation of inositol trisphosphate (IP3) and diacylglycerol (DAG). A transient flux in intracellular calcium promotes induction of calcineurin and NF-AT, as well as calmodulin (CaM)-dependent kinase and CREB. Increased DAG concentrations stimulate protein kinase C (PKC) and endosomal/lysosomal acidic sphingomyelinase (aSMase); while the aSMase pathway is dominant, both induce degradation of the NFκB inhibitor IκB as well as NFκB activation. By a poorly understood mechanism, IκB degradation may also be initiated through the MAPK (mitogen-activated protein kinase) cascade (not shown). (b) Growth factor receptor (depicted by ellipses) activation results in recruitment of Sos (not shown) to the plasma membrane, where it stimulates Ras, which recruits the serine/threonine kinase Raf to the plasma membrane. Once activated, Raf phosphorylates MEK kinase, which phosphorylates and activates MAPK and the transcription factor ELK (Ets-like protein, also known as p62 TCF1 (ternary complex factor 1)). ELK drives transcription from promoters with SRE elements, leading to synthesis of the transcription factors Fos and Jun, that form a transcription complex capable of activating AP1 sites. Seven transmembrane receptors also stimulate the MAPK pathway through βγ subunits, most probably through phosphoinositide 3-kinase γ (PI3Kγ; not shown).

of a number of different G-protein-coupled receptors. Recent advances in microscopic imaging technology, in conjunction with software permitting automated image recognition, provide a means to capture these events in a high-throughput mode.

Cell-based assays have significant advantages over *in vitro* assays. First, the starting material (the cell) self-replicates, avoiding the investment involved in preparing a purified target, in enzymically modifying the target to suit the screen, and so on. Second, the targets and readouts are ex-

Table 1

Reporter genes useful for cell-based high-throughput screening.

Reporter genes (source)	Advantages	Disadvantages	References
β-galactosidase (bacterial)	Well characterized; stable, inexpensive substrates; highly sensitive fluorescent or chemiluminescent substrates available; little interference from test compounds; simple readouts (readily automated)	Endogenous activity (mammalian cells); tetrameric (non-linear response at low concentration)	[23•,50]
Luciferase (firefly)	Dimeric; high specific activity; no endogenous activity (low background)	Requires addition of cofactor (luciferin) and presence of O_2 and ATP	[23•]
Alkaline phosphatase (human placental)	Secreted protein (avoids the need for membrane-permeable substrates); inexpensive colorimetric and highly sensitive luminescent assays available	Endogenous activity in some cell types; optimal at pH 9.8	[24,25]
β-lactamase (bacterial)	Monomeric; highly sensitive fluorogenic substrates described; no endogenous activity	Membrane-permeable fluorescent substrates not readily available	[27••]
GFP (jellyfish)	Monomeric; no substrate needed (no manipulations required for assay); no endogenous activity; multiple forms available	Relatively low specific activity	[26•,51,52]

amined in a biological context that more faithfully mimics the normal physiological situation. Third, cell-based assays can provide insights into bioavailability and cytotoxicity. Mammalian cells are expensive to culture and difficult to propagate in the automated systems used for HTS, however.

An alternative to mammalian cell based assays is to recapitulate the desired human physiological process in a micro-organism such as yeast [33]. For instance, signaling via human G-protein-coupled receptors has been reconstituted in yeast to yield a facile growth response or a reporter gene readout ([34,35]; Klein *et al.*, unpublished data). Similarly, mammalian ion channels have been coupled to growth response in yeast [36]. Also, protein–protein interactions, including RAS–RAF association [37] and tyrosine kinase receptor–ligand binding [38], have been faithfully reproduced using the yeast two-hybrid system. Finally, many mammalian transcription factors operate in yeast, including glucocorticoid receptor [39,40] and the retinoic acid receptor and retinoid X receptor families of receptors [41]. The ease and low cost of growing yeast, their ready genetic manipulation, and their resistance to solvents make yeast an attractive option for cell-based HTS.

Miniaturization

Several factors are fueling efforts to increase the speed of HTS and decrease the volume of individual reactions within an HTS format. Split-bead synthesis (see Note added in proof), or other similar approaches to combinatorial chemistry, dramatically increases the number of compounds that can be produced in a library but do so at the cost of quantity of material. In addition, the limited supply of existing compounds within chemical libraries of pharmaceutical companies, and the growing number of targets against which such compounds can be tested, motivate a frugal approach to use of those compounds. Finally, the reagent costs associated with HTS, when multiplied by the increasing number of assays per run, are becoming a significant cost of early stage drug discovery.

In response to these exigencies, a number of groups have begun to develop formats for very high density screening using very small assay volumes. One approach involves reducing the well size and increasing the density of the assay plate but retaining the overall assay format used in current 96-well based HTS. Densities of 6500 assays in a 10 cm array have been reported for cell-free enzyme based assays [42•] and for ligand binding in cell based assays [43••]. This approach of miniaturizing existing formats significantly increases the number of assays per plate and the overall throughput of the screen but is intrinsically limited by the physical constraints of delivering small volumes to wells, and of detecting responses in a sensitive and timely manner. Accordingly, novel formats have been developed that eschew the assay format based on wells. One approach uses glass chips containing microchannels in which reagents, target proteins and compounds are herded by electrokinetic flow controlled by electric potentials applied at the ends of the channels [44•]. A related approach attains high-throughput both of chemical synthesis and activity assessment by parallel arrays of three-dimensional channels in which flow is controlled by miniature hydrostatic actuators [45]. These approaches provide significant reduction in the volume of assays and a corresponding savings in reagent costs over conventional HTS [45]. In addition, with further development in parallel processing in multiple chips, the number of assays performed in a given period

of time can increase dramatically. This movement to miniaturization is likely to ensure that the initial stage of drug discovery identification of lead compounds will not be the rate-limiting step in finding new drugs.

Conclusions

The last decade has witnessed the emergence across the pharmaceutical industry of the 96-well-based, robotics-driven, high-throughput screening process as the primary tool for identifying active compounds in the first stage of drug discovery. This program has dictated the format of the assays that are used to assess the activities of targets—enzymes, receptors, transporters and so on—that underlie drug discovery in various therapeutic areas. A number of such formats—resonant energy transfer and fluorescent polarization spectroscopy in cell-based assays—have gained widespread acceptance and growing incorporation into high-throughput screening programs. The growing number of potential therapeutic targets, the increasing number of screenable compounds, the accelerating costs of screening and the increasing pressure to generate more lead compounds in a shorter time all conspire to render even the new approaches inadequate for meeting the anticipated throughput requirements, however. Thus, we are likely to witness a movement towards even greater screening throughput by miniaturization and increased reliance on robotics. Whether a new standard format for screening emerges in the near future, or whether a variety of formats are pursued concurrently remains to be seen. Nonetheless, we can anticipate that the exigencies of drug screening will motivate a continued application of state-of-the-art technologies to the process of high-throughput screening.

N te added in proof

For a reference describing split-bead synthesis, see [53].

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Udenfriend S, Gerber LD, Brink L, Spector S: Scintillation proximity assay: a sensitive and continuous isotropic method for monitoring ligand/receptor and antigen/antibody interactions. *Anal Biochem* 1985, 161:494-500.

2. Cook N: Scintillation proximity assay: a versatile high-throughput screening technology. *Drug Discov Today* 1996, 1:287.

3. Lemor C, Saiki A: Scintillation proximity assay for human DNA topoisomerase I using recombinant biotinyl-fusion protein produced in baculovirus-infected insect cells. *Anal Biochem* 1996, 240:185-196.

4. Mathis G: Rare earth cryptates and homogeneous fluoroimmunoassays with human sera. *Clin Chem* 1993, 39:1953-1959.

5. Mathis G: Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin Chem* 1995, 41:1391-1397.

6. • Braunwalder A, Yarwood D, Sills M, Lipson K: Measurement of the protein tyrosine kinase activity of c-src using time-resolved fluorometry of europium chelates. *Anal Biochem* 1996, 238:159-164.

The authors describe an assay method to evaluate the activity of protein tyrosine kinases that uses europium chelate-labeled anti-phosphotyrosine antibodies to detect phosphate transfer to a polymeric substrate coated onto microtiter plate wells. Using time-resolved, dissociation-enhanced fluorescence increased sensitivity and reduced interference from test compounds.

7. • Gaarde W, Hunter T, Brady H, Murray B, Goldman M: Development of a nonradioactive, time-resolved fluorescence assay for the measurement of jun n-terminal kinase activity. *J Biomol Screen* 1997, 2:213-223.

The authors describe a nonradioactive, high-throughput, time-resolved fluorescence assay for jun kinase (JNK) activity using europium-labeled antibody that is specific for amino-terminally phosphorylated c-jun. The optimized europium-based assay is approximately 15-fold more sensitive than a similar ^{32}P-based JNK assay.

8. • Mitra R, Silva C, Youvan D: Fluorescence resonance energy transfer between blue-emitting and red- shifted excitation derivatives of the green fluorescent protein. *Gene* 1996, 173:13-17.

The authors report fluorescent resonance energy transfer (FRET) between two linked variants of the green fluorescent protein, one of which is fused to the amino terminus of a flexible polypeptide linker containing a Factor X a protease cleavage site while the second of which is fused to the carboxy terminus of the polypeptide. Cleavage of the peptide linker with Factor Xa yields a marked decrease in energy transfer, making this a viable homogeneous assay format for proteases.

9. Aucouturier P, Preudhomme JL, Lubochinsky B: Fluorescence polarization immunoassay of estradiol. *Diagn Clin Immunol* 1983, 1:310-314.

10. Eremin SA, Gallacher G, Lotey H, Smith DS, Landon J: Single-reagent polarization fluoroimmunoassay of methamphetamine in urine. *Clin Chem* 1987, 33:1903-1906.

11. Wei A-P, Herron JN: Use of synthetic peptides as tracer antigens in fluorescence polarization immunoassays of high molecular weight analytes. *Anal Chem* 1993, 65:3372-3377.

12. Murakami A, Nakaura M, Nakatsuji Y, Nagahara S, Tran-Cong O, Makino K: Fluorescent-labeled oligonucleotide probes: detection of hybrid formation in solution by fluorescence polarization spectroscopy. *Nucl Acids Res* 1991, 19:4097-4102.

13. Jolley Consulting and Research Inc on the World Wide Web; http://www.jolley.com/.

14. • Seethala R, Menzel R: A homogeneous, fluorescence polarization assay for src-family tyrosine kinases. *Anal Biochem* 1997, 253:210-218.

The authors describe a homogeneous assay for the src kinase family member, Lck, that consists of a fluoresceinylated peptide substrate for the kinase and an anti-phosphotyrosine antibody.

15. • Lynch B, Lolacono K, Tiong C, Adams S, MacNeil I: A fluorescence polarization based Src-SH2 binding assay. *Anal Biochem* 1997, 247:77-82.

The authors describe an assay to detect compounds that interfere with the binding of an SH2 domain to its phosphotyrosine peptide target. The assay consists of the src SH2 domain and a fluoresceinylated, phosphotyrosine-containing hexapeptide to which the src SH2 domain binds. Compounds that interfere with this interaction are detected by a decrease in fluorescence polarization.

16. • Wu P, Brasseur M, Schindler U: A high-throughput STAT binding assay using fluorescence polarization. *Anal Biochem* 1997, 249:29-36.

The authors describe an assay to detect compounds that interfere with the interaction between STAT1, a transcription factor that is activated upon γ-interferon binding to its receptor, and the phosphotyrosine-containing peptide derived from γ-interferon receptor with which it interacts. Binding is evaluated using fluorescence polarization.

17. Schade S, Jolley M, Sarazar B, Simonson L: BODIPY-alpha-casein, a pH-independent protein substrate for protease assays using fluorescence polarization. *Anal Biochem* 1996, 243:1-7.

18. • Levine L, Michener M, Toth M, Holwerda B: Measurement of specific protease activity utilizing fluorescence polarization. *Anal Biochem* 1997, 247:83-88.

The authors describe a homogeneous fluorescence polarization assay to measure proteolytic cleavage of the peptide substrate for a protease. The peptide substrate was derivatized by biotinylation of the amino terminus and labeled with a fluorescein derivative at the carboxy terminus. Incubation of this substrate with protease and subsequent addition of avidin produced a polarization signal that was proportional to the relative amounts of cleaved

and uncleaved substrate. The authors demonstrated that the assay does not suffer from interference due to the presence of light absorptive compounds in the mixture.

19. Eigen M, Rigler R: Sorting single molecules: application to diagnostics and evolutionary biotechnology. *Proc Natl Acad Sci USA* 1994, 91:5740-5747.

20. Sterrer S, Henco K: Fluorescence correlation spectroscopy (FCS)-a highly sensitive method to analyze drug/target interactions. *J Recept Signal Transduct Res* 1997, 17:511-520.

21. Dhundale A, Goddard C: Reporter assays in the high-throughput screening laboratory: A rapid and robust first look? *J Biomol Screen* 1996, 1:115-118.

22. Sato CM, Ignar DM: Selection of an optimal reporter gene for cell-based high throughput screening assays. *J Biomol Screen* 1997, 2:7-8.

23. • Martin C, Wight P, Dobretsova A, Bronstein I: Dual luminescence-based reporter gene assay for luciferase and β-galactosidase. *Biotechniques* 1996, 21:520-524.
The authors describe highly sensitive chemiluminescent assays for firefly luciferase and β-galactosidase, which can detect as little as 2 fg of luciferase and 8 fg of β-galactosidase, has a dynamic range over seven orders of magnitude of enzyme concentration and both assays can be performed on the same sample.

24. Bronstein I, Fortin J, Voyta J, Juo R, Edwards B, Olesen C, Ljam N, Kricka L: Chemiluminescent reporter gene assays: sensitive detection of the GUS and SEAP gene products. *Biotechniques* 1994, 17:172-177.

25. Bronstein I, Martin C, Fortin J, Olesen C, Voyta J: Chemiluminescence: sensitive detection technology for reporter gene assays. *Clin Chem* 1996, 42:1542-1546.

26. • Misteli T, Spector D: Applications of the green fluorescent protein in cell biology and biotechnology. *Nat Biotechnol* 1997, 15:961-964.
The authors present an overview of some of the major applications of an autofluorescent protein, the green fluorescent protein, namely its use in protein tagging, in monitoring gene expression and in a variety of biological screens.

27. •• Zlokarnik G, Negulescu P, Knapp T, Mere L, Burres N, Feng L, Whitney M, Roemer K *et al.*: Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. *Science* 1998, 279:84-88.
In this chemical and biological tour de force, the authors describe a substrate for β-lactamase that can be readily loaded into living cells and whose hydrolysis by β-lactamase causes a cell-restricted, fluorescense color shift from green to blue that can be detected by eye. The assay allows analysis of gene expression in individual mammalian cells and enables clonal selection by flow cytometry.

28. • Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, Milligan G, Lee M *et al.*: A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Anal Biochem* 1997, 252:115-126.
The authors report that transient transvection of a chinese hamster ovary cell line with the genes for aposequorin, $G_{\alpha 16}$ and any of a number of seven transmembrane receptors resulted in a large, concentration-dependent agonist-mediated luminescent response. The authors suggest that this approach provides a basis for a generic mammalian cell microplate assay for the assessment of agonist action at virtually any G-protein-coupled receptor, including orphan receptors for which the physiological signal transduction mechanism may be unknown.

29. •• Miyawaki A, Llopis J, Heim R, McCaffery J, Adams J, Ikura M, Tsien R: Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature* 1997, 388:882-887.
The authors describe the construction and use of novel fluorescent indicators for intracellular Ca^{2+}, which consist of tandem fusions of a blue- or cyan-emitting mutant of the green fluorescent protein (GFP), calmodulin, the calmodulin-binding peptide M13 and an enhanced green- or yellow- emitting GFP. Binding of Ca^{2+} makes calmodulin wrap around the M13 domain, increasing the fluorescence resonance energy transfer (FRET) between the flanking GFPs. The authors can detect free Ca^{2+} dynamics in the cytosol, nucleus and endoplasmic reticulum of single HeLa cells transfected with complementary DNAs encoding chimeras bearing appropriate localization signals.

30. Lenormand P, Sardet C, Pages G, L'Allemain G, Brunet A, Pouysségur J: Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J Cell Biol* 1993, 122:1079-1088.

31. Gonzalez FA, Seth A, Raden DL, Bowman DS, Fay FS, Davis RJ: Serum-induced translocation of mitogen activated protein kinase to the cell surface ruffling membrane and the nucleus. *J Cell Biol* 1993, 122:1089-1101.

32. Barak L, Ferguson S, Zhang J, Caron M: A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* 1997, 272:27497-27500.
The authors describe using a β-arrestin2/green fluorescent protein conjugate to obtain a real-time and single cell based assay to monitor G protein-coupled receptor (GPCR) activation and GPCR–G-protein-coupled receptor kinase or GPCR–arrestin interactions. They show, by confocal microscopy, that the β-arrestin conjugate translocates to the plasma membrane in response to activation of any one of more than 15 different ligand-activated GPCRs, suggesting that β-arrestin binding of an activated receptor is a convergent step of GPCR signaling.

33. Klein RD, Geary TG: Recombinant microorganisms as tools for high throughput screening for nonantibiotic compounds. *J Biomol Screen* 1997, 2:41-49.

34. King K, Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ: Control of yeast mating signal transduction by a mammalian b2-adrenergic receptor and Gsa subunit. *Science* 1990, 250:121-123.

35. Price LA, Kajkowski EM, Hadcock JR, Ozenberger BA, Pausch MH: Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. *Mol Cell Biol* 1995, 15:6188-6195.

36. Tang W, Rudnudin A, Yang WP, Shaw SY, Knickerbocker A, Kurtz S: Functional expression of a vertebrate inwardly rectifying K+ channel in yeast. *Mol Biol Cell* 1995, 6:1231-1240.

37. van Aelst L, Barr M, Marcus S, Polverino A, Wigler M: Complex formation between RAS and RAF and other protein kinases. *Proc Natl Acad Sci USA* 1993, 90:6213-6217.

38. Ozenberger BA, Young KH: Functional interaction of ligands and receptors of the hematopoietic superfamily in yeast. *Mol Endocrinol* 1995, 9:1321-1329.

39. Schena M, Yamamoto KR: Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. *Science* 1988, 241:241-244.

40. Krall A, Bohen SP, Yamamoto KR: LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. *Proc Natl Acad Sci USA* 1995, 92:4701-4705.

41. Hall BL, Smit-McBride Z, Privalsky ML: Reconstitution of the retinoid X receptor function and combinatorial regulation of other nuclear hormone receptors in the yeast *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 1993, 90:6929-6933.

42. • Schullek J, Butler J, Ni Z, Chen D, Yuan Z: A high-density screening format for encoded combinatorial libraries: assay miniaturization and its application to enzymatic reactions. *Anal Biochem* 1997, 246:20-29.
The authors describe a novel, miniaturized high-throughput screening format for assaying combinatorial libraries generated on beads, in which compounds are photolytically released from beads into a high-density well array (>6,500 wells within a standard 96-well microtiter plate footprint) with well volumes as low as 0.37 nl. Use of the format to detect inhibitors of a member of the matrix metalloproteinase superfamily is described.

43. •• You A, Jackman R, Whitesides G, Schreiber S: A miniaturized arrayed assay format for detecting small molecule-protein interactions in cells. *Chem Biol* 1997, 4:969-975.
The authors describe a miniaturized cell-based technique for the screening of ligands prepared by split-pool synthesis, in which spatially defined droplets with uniform volumes of approximately 50–150 nl are arrayed on plastic devices prepared using a combination of photolithography and polymer molding. Using this microtechnology, approximately 6,500 assays, using either yeast cells or mammalian tissue culture, could be performed within the dimensions of a standard 10 cm petri dish.

44. • Hadd A, Raymond D, Halliwell J, Jacobson S, Ramsey J: Microchip device for performing enzyme assays. *Anal Chem* 1997, 69:3407-3412.
The authors describe a novel, miniaturized enzyme assay format, performed within a microfabricated channel network in which precise concentrations of substrate, enzyme and inhibitor were mixed in nanoliter volumes using electrokinetic flow. Reaction kinetics for β-galactosidase obtained by this format were identical to those obtained by conventional assays but required four orders of magnitude less material to do so.

45. Rogers M: High-throughput screening: miniaturization of high-throughput fluorescence detection for the determination of a few β-galactosidase molecules. *Drug Discov Today* 1997, 2:306.

46. Eder J: Tumor necrosis factor α and interleukin 1 signaling: do MAPKK kinases connect it all? *Trends Pharmacol* 1997, 320:319-322.

47. Hill CS, Treisman R: Transcriptional regulation by extracellular signals: mechanism and specificity. *Cell* 1995, 80:199-211.

48. Premack BA, Schall TJ: Chemokine receptors: gateway to inflammation and infection. *Nature Med* 1996, 11:1174-1178.

49. Schutze S, Wolgmann K, Machleidt T, Kronke M: TNF-induced activation of NF-kB. *Immunobiology* 1995, 193:193-203.

50. Craig D, Arriaga E, Banks P, Zhang Y, Renborg A, Palcic M, Dovichi N: Fluorescence-based enzymatic assay by capillary electrophoresis laser-induced. *Anal Biochem* 1995, 226:147-153.

51. Anderson M, Tjioe I, Lorincz M, Parks D, Herzenberg L, Nolan G, Herzenberg L: Simultaneous fluorescence-activated cell sorter analysis of two distinct transcriptional elements within a single cell using engineered green fluorescent proteins. *Proc Natl Acad Sci USA* 1996, 93:8508-8511.

52. Heim R, Tsien R: Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr Biol* 1996, 6:178-182.

53. Burbaum JJ, Ohlmeyer MH, Reader JC, Henderson I, Dillard LW, Randle TL, Sigal NH, Chelsky D, Baldwin JJ: A paradigm for drug discovery employing encoded combinatorial libraries. *Proc Natl Acad Sci USA* 1995, 92:6027-6031.

Appendix G

lalign output for SEQ ID NO:5 vs. SEQ ID NO: 36

[ISREC-Server] Date: Mon Jan 14 20:59:50 MET 2002

---

reset matrix file to /export/molbio/share/fasta2/blosum80.mat ./wwwtmp/lalign/.24621.1.seq : 764 aa

```
ALIGN calculates a global alignment of two sequences
 version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17
SEQ ID NO:5                                              764 aa vs.
SEQ ID NO: 36                                            725 aa
scoring matrix: /export/molbio/share/fasta2/blosum80.mat, gap penalties: -14/-4
88.3% identity;         Global alignment score: 3493

               10        20        30        40        50        60
./wwwt MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
               10        20        30        40        50        60

               70        80        90       100       110       120
./wwwt RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
               70        80        90       100       110       120

              130       140       150       160       170       180
./wwwt MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
              130       140       150       160       170       180

              190       200       210       220       230       240
./wwwt LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
              190       200       210       220       230       240

              250       260       270       280       290       300
./wwwt TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
              250       260       270       280       290       300

              310        320        330        340       350
./wwwt AAKEGKIEIFRHILQR-EFSGLSH-LSRKFTEW-CYGPVRVSLYDLASVDSCEENSVLEI
       ::::::: ::::::   .:::::   . ::::::   :::::::::::::::::::::: :
SEQ    AAKEGKIGIFRHILASGKFSGLKPPFPRKFTEWWLMGPVRVSLYDLASVDSCEENSVLGI
              310       320       330       340       350       360

       360       370       380       390       400       410
./wwwt IAFHCKSPHRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKK
       :::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    IAFHCKSPDRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKK
              370       380       390       400       410       420

       420       430       440       450       460       470
./wwwt QAAPHLKAEVGNSMLLTGHILILLGGIYLLVGQLWYFWRRHVFIWISFIDSYFEILFLFQ
       :::::::::::::::::::::::::::::::: : ::     ::: ::::::::::::
```

```
SEQ     QAAPHLKAEVGNSMLLTGHILILLGGIYLLVGQKWKFW-----IWI-FIDSYFEILFLFP
               430       440       450             460       470

        480       490       500       510       520       530
./wwwt ALLTVVSQVLCFLAIEWYLPLLVSALVLGWLNLLYYTRGFQHTGIYSVMIQKVILRDLLR
        .   ::    :  :      :   ::.  ::       . .:   .
SEQ     GH-RVVPAPACVCA---GAGLAEPALLYTWL------PAHRHLQCHD-------------
            480           490       500             510

        540       550       560       570       580       590
./wwwt FLLIYLVFLFGFAVALVSLSQEAWRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEAS
                     :::::::. ::::::::::::::::::::::::::::::::::::
SEQ     ------------PEALVSLSQD-WRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEAS
                           520       530       540       550

        600       610       620       630       640       650
./wwwt LELFKFTIGMGELAFQEQLHFRGMVLLLLLAYVLLTYILLLNMLIALMSETVNSVATDSW
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ     LELFKFTIGMGELAFQEQLHFRGMVLLLLLAYVLLTYILLLNMLIALMSETVNSVATDSW
       560       570       580       590       600       610

        660       670       680       690       700       710
./wwwt SIWKLQKAISVLEMENGYWWCRKKQRAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ     SIWKLQKAISVLEMENGYWWCRKKQRAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQT
       620       630       640       650       660       670

        720       730       740       750       760
./wwwt LPTLCEDPSGAGVPRTLENPVLASPPKEDEDGASEENYVPVQLLQSN
        :::::::::::::::::::::::::::::::::::::::::::::
SEQ     LPTLCEDPSGAGVPRTLENPVLASPPKEDEDGASEENYVPVQLLQSN
       680       690       700       710       720
```

Back to ISREC bioinformatics group home page

Appendix H

lalign output for SEQ ID NO:5 vs. SEQ ID NO: 2

[ISREC-Server] Date: Mon Jan 14 19:59:06 MET 2002

---

reset matrix file to /export/molbio/share/fasta2/blosum80.mat ./wwwtmp/lalign/.9148.1.seq : 764 aa

```
ALIGN calculates a global alignment of two sequences
 version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17
SEQ ID NO:5                                              764 aa vs.
SEQ ID NO: 2                                             763 aa
scoring matrix: /export/molbio/share/fasta2/blosum80.mat, gap penalties: -14/-4
99.9% identity;         Global alignment score: 4297

               10        20        30        40        50        60
./wwwt MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
               10        20        30        40        50        60

               70        80        90       100       110       120
./wwwt RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
               70        80        90       100       110       120

              130       140       150       160       170       180
./wwwt MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
              130       140       150       160       170       180

              190       200       210       220       230       240
./wwwt LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
              190       200       210       220       230       240

              250       260       270       280       290       300
./wwwt TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
              250       260       270       280       290       300

              310       320       330       340       350       360
./wwwt AAKEGKIEIFRHILQREFSGLSHLSRKFTEWCYGPVRVSLYDLASVDSCEENSVLEIIAF
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    AAKEGKIEIFRHILQREFSGLSHLSRKFTEWCYGPVRVSLYDLASVDSCEENSVLEIIAF
              310       320       330       340       350       360

              370       380       390       400       410       420
./wwwt HCKSPHRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKKQAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
SEQ    HCKSPHRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKK-AA
              370       380       390       400       410

              430       440       450       460       470       480
./wwwt PHLKAEVGNSMLLTGHILILLGGIYLLVGQLWYFWRRHVFIWISFIDSYFEILFLFQALL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
```

```
SEQ    PHLKAEVGNSMLLTGHILILLGGIYLLVGQLWYFWRRHVFIWISFIDSYFEILFLFQALL
     420       430       440       450       460       470

               490       500       510       520       530       540
./wwwt TVVSQVLCFLAIEWYLPLLVSALVLGWLNLLYYTRGFQHTGIYSVMIQKVILRDLLRFLL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    TVVSQVLCFLAIEWYLPLLVSALVLGWLNLLYYTRGFQHTGIYSVMIQKVILRDLLRFLL
     480       490       500       510       520       530

               550       560       570       580       590       600
./wwwt IYLVFLFGFAVALVSLSQEAWRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEASLEL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    IYLVFLFGFAVALVSLSQEAWRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEASLEL
     540       550       560       570       580       590

               610       620       630       640       650       660
./wwwt FKFTIGMGELAFQEQLHFRGMVLLLLLAYVLLTYILLLNMLIALMSETVNSVATDSWSIW
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    FKFTIGMGELAFQEQLHFRGMVLLLLLAYVLLTYILLLNMLIALMSETVNSVATDSWSIW
     600       610       620       630       640       650

               670       680       690       700       710       720
./wwwt KLQKAISVLEMENGYWWCRKKQRAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQTLPT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    KLQKAISVLEMENGYWWCRKKQRAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQTLPT
     660       670       680       690       700       710

               730       740       750       760
./wwwt LCEDPSGAGVPRTLENPVLASPPKEDEDGASEENYVPVQLLQSN
       ::::::::::::::::::::::::::::::::::::::::::::
SEQ    LCEDPSGAGVPRTLENPVLASPPKEDEDGASEENYVPVQLLQSN
     720       730       740       750       760
```

Back to ISREC bioinformatics group home page

Appendix I

lalign output for SEQ ID NO:20 vs. SEQ ID NO:36

[ISREC-Server] Date: Tue Jan 29 16:25:59 MET 2002

./wwwtmp/lalign/.651.1.seq : 630 aa

```
ALIGN calculates a global alignment of two sequences
 version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17
SEQ ID NO:20                                             630 aa vs.
SEQ ID NO:36                                             725 aa
scoring matrix: BLOSUM50, gap penalties: -14/-4
78.2% identity;         Global alignment score: 3318

               10        20        30        40        50        60
./wwwt MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
               10        20        30        40        50        60

               70        80        90       100       110       120
./wwwt RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
               70        80        90       100       110       120

              130       140       150       160       170       180
./wwwt MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
              130       140       150       160       170       180

              190       200       210       220       230       240
./wwwt LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
              190       200       210       220       230       240

              250       260       270       280       290       300
./wwwt TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
              250       260       270       280       290       300

              310        320        330        340       350
./wwwt AAKEGKIEIFRHILQR-EFSGLSH-LSRKFTEW-CYGPVRVSLYDLASVDSCEENSVLEI
       ::::::: ::::::   .:::::.  . ::::::  .:::::::::::::::::::::: :
SEQ    AAKEGKIGIFRHILASGKFSGLKPPFPRKFTEWWLMGPVRVSLYDLASVDSCEENSVLGI
              310       320       330       340       350       360

       360       370       380       390       400       410
./wwwt IAFHCKSPHRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKK
       :::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    IAFHCKSPDRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKK
              370       380       390       400       410       420

       420       430       440       450       460       470
./wwwt QAAPHLKAEVGNSMLLTGHILILLGGIYLLVGQLWYFWRRHVFIWISFIDSYFEILFLFQ
       :::::::::::::::::::::::::::::::: : ::     ::: ::::::::::::
```

```
SEQ    QAAPHLKAEVGNSMLLTGHILILLGGIYLLVGQKWKFW-----IWI-FIDSYFEILFLFP
              430       440       450             460       470

       480                 490                 500
./wwwt ALLTVVSQV-LCF--------LAIEW---------YLPLLVSALVLGW------------
       .  .:  .  .  .:          :   :          .  :   .  .:    :
SEQ    GHRVVPAPACVCAGAGLAEPALLYTWLPAHRHLQCHDPEALVSLSQDWRPEAPTGPNATE
          480       490       500       510       520       530

                                510
./wwwt -------------------------LNLLYYTRG-----FQH-------------------
                                :.:. .: :     ::.
SEQ    SVQPMEGQEDEGNGAQYRGILEASLELFKFTIGMGELAFQEQLHFRGMVLLLLLAYVLLT
          540       550       560       570       580       590

                           520       530       540       550
./wwwt ---------------------TGIYSVMIQKKAISVLEMENGYWWCRKKQRAGVMLTVGTK
                            :  .:.   .:::::::::::::::::::::::::::::
SEQ    YILLLNMLIALMSETVNSVATDSWSIWKLQKAISVLEMENGYWWCRKKQRAGVMLTVGTK
          600       610       620       630       640       650

     560       570       580       590       600       610
./wwwt PDGSPDERWCFRVEEVNWASWEQTLPTLCEDPSGAGVPRTLENPVLASPPKEDEDGASEE
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    PDGSPDERWCFRVEEVNWASWEQTLPTLCEDPSGAGVPRTLENPVLASPPKEDEDGASEE
          660       670       680       690       700       710

     620       630
./wwwt NYVPVQLLQSN
       ::::::::::::
SEQ    NYVPVQLLQSN
          720
```

Back to ISREC bioinformatics group home page

Appendix J

lalign output for SEQ ID NO:20 vs. SEQ ID NO:2

[ISREC-Server] Date: Tue Jan 29 16:26:37 MET 2002

./wwwtmp/lalign/.1391.1.seq : 630 aa

```
ALIGN calculates a global alignment of two sequences
 version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17
SEQ ID NO:20                                             630 aa vs.
SEQ ID NO:2                                              763 aa
scoring matrix: BLOSUM50, gap penalties: -14/-4
82.3% identity;         Global alignment score: 3710

               10        20        30        40        50        60
./wwwt MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MTSPSSSPVFRLETLDGGQEDGSEADRGKLDFGSGLPPMESQFQGEDRKFAPQIRVNLNY
               10        20        30        40        50        60

               70        80        90       100       110       120
./wwwt RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    RKGTGASQPDPNRFDRDRLFNAVSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCL
               70        80        90       100       110       120

              130       140       150       160       170       180
./wwwt MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    MKAVLNLKDGVNACILPLLQIDRDSGNPQPLVNAQCTDDYYRGHSALHIAIEKRSLQCVK
              130       140       150       160       170       180

              190       200       210       220       230       240
./wwwt LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    LLVENGANVHARACGRFFQKGQGTCFYFGELPLSLAACTKQWDVVSYLLENPHQPASLQA
              190       200       210       220       230       240

              250       260       270       280       290       300
./wwwt TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    TDSQGNTVLHALVMISDNSAENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKL
              250       260       270       280       290       300

              310       320       330       340       350       360
./wwwt AAKEGKIEIFRHILQREFSGLSHLSRKFTEWCYGPVRVSLYDLASVDSCEENSVLEIIAF
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    AAKEGKIEIFRHILQREFSGLSHLSRKFTEWCYGPVRVSLYDLASVDSCEENSVLEIIAF
              310       320       330       340       350       360

              370       380       390       400       410       420
./wwwt HCKSPHRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKKQAA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
SEQ    HCKSPHRHRMVVLEPLNKLLQAKWDLLIPKFFLNFLCNLIYMFIFTAVAYHQPTLKK-AA
              370       380       390       400       410

              430       440       450       460       470       480
./wwwt PHLKAEVGNSMLLTGHILILLGGIYLLVGQLWYFWRRHVFIWISFIDSYFEILFLFQALL
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
```

```
SEQ    PHLKAEVGNSMLLTGHILILLGGIYLLVGQLWYFWRRHVFIWISFIDSYFEILFLFQALL
     420       430       440       450       460       470

               490       500       510       520
./wwwt TVVSQVLCFLAIEWYLPLLVSALVLGWLNLLYYTRGFQHTGIYSVMIQK----------
       ::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    TVVSQVLCFLAIEWYLPLLVSALVLGWLNLLYYTRGFQHTGIYSVMIQKVILRDLLRFLL
     480       490       500       510       520       530


./wwwt ----------------------------------------------------------

SEQ    IYLVFLFGFAVALVSLSQEAWRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEASLEL
     540       550       560       570       580       590


./wwwt ----------------------------------------------------------

SEQ    FKFTIGMGELAFQEQLHFRGMVLLLLLAYVLLTYILLLNMLIALMSETVNSVATDSWSIW
     600       610       620       630       640       650

        530       540       550       560       570       580
./wwwt ---KAISVLEMENGYWWCRKKQRAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQTLPT
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SEQ    KLQKAISVLEMENGYWWCRKKQRAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQTLPT
     660       670       680       690       700       710

        590       600       610       620       630
./wwwt LCEDPSGAGVPRTLENPVLASPPKEDEDGASEENYVPVQLLQSN
       ::::::::::::::::::::::::::::::::::::::::::::
SEQ    LCEDPSGAGVPRTLENPVLASPPKEDEDGASEENYVPVQLLQSN
     720       730       740       750       760
```

Back to ISREC bioinformatics group home page